REMARKS/ARGUMENTS

Claims 47-70 are active in this application.

Support for the definition of the cell as a "mammalian cell" is found throughout the specification, e.g., on page 47, lines 10-11.

Claims 58-70 find support in Claims 47-57 where the difference is the presence of the HIV-1 promoter rather than the HIV-2 promoter. This finds support, e.g., in Table 5 on page 67 and Figures 8 and 9.

Support for the CMV promoter in Claims 58 and 70 is found in Table 3 and Figure 9. The fact that the CMV promoter described in the application inherently is responsive or induced by heat is discussed in the attached manuscript of Dammeyer et al (also including named inventors Tsang and Harris) where in figure 2 of this paper, the data showed that the CMV promoter construct, pCMV-GM-CSF, has significantly higher levels of expression after heating at 42C for 30 minutes (black bars) as compared to the unheated controls (white bars) at 24 hours, 48 hours and 72 hours after heating. See also, the discussion section of the manuscript at page 12: "constitutive GM-CSF expression by the CMV promoter in pCMV-GM-CSF was also responsive to heat treatment with expression levels close to those achieved with the HSP70B promoter in pAD-Hot-GM-CSF. A similar stress-induced increase in transcription with the CMV promoter was previously observed (39, 40)."

No new matter is believed to have been added by these amendments.

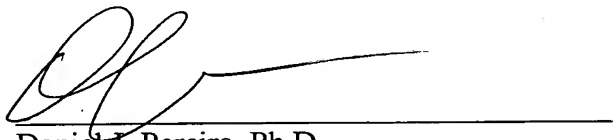
Applicants thank the Examiner for recognizing that methods of expressing in a mammalian cell are enabled (see page 2 of the Office Action). As amended herein, the claims are directed to the expression of the selected polynucleotide in a mammalian cell. Accordingly, withdrawal of the rejection under 35 USC 112, first paragraph is requested.

To the rejections under the doctrine of obviousness-type double patenting in view of U.S. patent no. 6,709,858 ad copending applications 10/108,486 and 10/152,577, a terminal disclaimer is filed with this amendment. Withdrawal of the rejections is requested.

Finally, Applicants request a Notice of Allowance for all pending claims.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.
Norman F. Oblon



Daniel J. Pereira, Ph.D.
Registration No. 45,518

Customer Number
22850

Tel: (703) 413-3000
Fax: (703) 413 -2220
(OSMMN 06/04)

Heat-inducible amplifier vector for high-level expression of granulocyte-macrophage colony-stimulating factor

Pascal Dammeyer[1, 2], Melba C. Jaramillo[1], Brian L. Pipes[1], Tom C. Tsang[1] and David T. Harris[1]

[1]*Department of Microbiology and Immunology, University of Arizona, Tucson, AZ 85724-5049, USA*

[2]*Present Address: Microbiology and Tumor Biology Center, Karolinska Institutet, SE-17177 Stockholm, Sweden*

Correspondence: Pascal Dammeyer
Karolinska Institutet
Box 280, Microbiology and Tumor Biology Center
SE-17177 Stockholm, Sweden
Phone: +46-(0)8-524-86091. Fax: +46-(0)8-330498
E-mail: Pascal.Dammeyer@ki.se

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Abstract

In cytokine immunotherapy of cancer it is critical to deliver sufficiently high local cytokine concentrations in order to reach the therapeutic threshold needed for clinical efficacy. Simultaneously, for optimal clinical safety adverse effects caused by high systemic cytokine levels must be minimized. One of the most promising anticancer therapeutic cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF), has elicited antitumor immune responses in animal studies and clinical trials. However, the clinical efficacy has been limited, with local GM-CSF levels being therapeutically insufficient and systemic toxicity being a limiting factor. To address these problems we have developed a novel GM-CSF expression vector, pAD-HotAmp-GM-CSF, which can provide high levels of GM-CSF expression, and induction of cytokine expression to limited tissue areas. This expression system combines inducible and amplifying elements in a single multigenic construct. The first transcriptional unit contains the inducible element, the heat shock protein 70B (HSP70B) promoter that regulates expression of the transcription-activating factor tat. Upon the binding of tat to the second promoter, the HIV2 long terminal repeat amplifies downstream gene expression of the therapeutic cytokine GM-CSF. Moderate hyperthermia at 42°C for 30 minutes induced GM-CSF expression in pAD-HotAmp-GM-CSF that was over 2.5 and 2.8 fold higher than levels reached with HSP70B promoter alone and the prototypical human cytomegalovirus promoter. Thus, the inducible amplifier vector, pAD-HotAmp-GM-CSF, represents a novel system for regulated and enhanced GM-CSF expression, which enables both greater efficacy and safety in cytokine immunotherapy of cancer.

Introduction

Strategies for the treatment of cancer include immunotherapy with recombinant cytokines, where the administration of single, or combinations, of cytokines can enhance a host's antitumor responses (1). Therefore, the composition of cytokines in the tumor microenvironment is critical (2). Systemic cytokine administration, in doses high enough to provide therapeutic tumor microenvironment levels, has proven toxic to the patient (3, 4). Gene therapy with therapeutic cytokine genes provides an alternative strategy (1, 5-7) if the transfection or expression is local. Thus, systemic adverse effects can be circumvented. However, locally targeted *in vivo* transfection still remains a difficult task (1, 8). Yet gene therapy with therapeutic cytokine genes shows great promise, as genetically engineered tumor cells secreting cytokines are often rejected quickly in mice that have additionally developed immune memory against subsequent challenges, even with poorly immunogenic tumors (8). Among the immunotherapy cytokines under investigation, granulocyte-macrophage colony-stimulating factor (GM-CSF) has demonstrated potential efficacy in modulating the immune system to combat cancer. GM-CSF is a factor with broad effects on progenitor cells of the myeloid lineage for proliferation and differentiation (9).

In animal studies, GM-CSF cancer immunotherapy has been extensively researched. Vaccination with GM-CSF-secreting tumor cells has induced either complete rejection or development of tumors with smaller volumes compared to the parental tumors (10, 11), has induced significant decrease or inhibition of subcutaneous tumor growth (7, 10), has prolonged survival times (7, 11), and has induced protection from subsequent tumor challenges of the treated mice (6). In multiple murine models for head and neck squamous cell carcinoma (12) and for metastatic melanoma (13), potent and specific protection, and in some cases long-lasting antitumor immunity against subsequent tumor challenges could be stimulated through this vaccination strategy. The response effect of tumor growth inhibition

to GM-CSF strongly correlates with dose-dependence (10, 14). Continuous high concentrations of the cytokine in the tumor microenvironment seem to be essential (6, 7, 15).

However, cytokine immunotherapy animal studies have generally yielded few successful clinical responses (4, 16-18) possibly because the required cytokine levels for humans were not achieved. A study using GM-CSF operating as an adjuvant in vaccination showed limited clinical benefits (10). Solitary T-cell responses may be incomplete to produce significant anticancer objective responses in patients with an advanced state of disease (14). In one study of intradermal injection of GM-CSF-transfected melanoma cells, one patient exhibited positive delayed-type hypersensitivity, a significant histological inflammatory response, and developed clinically stable disease (19). In this patient GM-CSF production of greater than 30 ng per 10^6 cells per 24 h *in vitro* was observed, contrasted with the majority of the remaining patients who stayed below 3 ng of GM-CSF production. Promising clinical antitumor immune responses to vaccination with irradiated tumor cells, engineered to secrete GM-CSF, were detected in some of the treated patients suffering from kidney or prostate cancer (20), non-small-cell lung cancer (NSCLC) (13, 21), and metastatic melanoma (22, 23). A dose-related antitumor response to GM-CSF-secreting vaccines was observed (20, 21). The sporadic potent clinical outcome in these patients supports the necessity of high local GM-CSF expression in order to reach the therapeutic threshold needed for antitumor immunotherapy.

In general the problem of expression of sufficient local levels of therapeutic antitumor cytokine has remained unsolved (1, 8). Strategies to improve cytokine immunotherapy vectors have focused on improving cytokine expression using different promoters. It has been generally concluded that the cytomegalovirus (CMV) promoter is the strongest currently available promoter (24-27). Yet, further improvements in GM-CSF gene expression will be necessary in order to consistently meet the required therapeutic threshold (28). To achieve expression levels of GM-CSF higher than those possible using the CMV

promoter, we have constructed a novel vector that is able to effectively "amplify" the gene expression of any promoter, including CMV. The amplifier design applied in this study involves two interacting genes. The first gene, encoding the transcription-activator protein tat, derived from the human immunodeficiency virus (HIV), can activate the HIV2 long terminal repeat (LTR) promoter controlling the second gene, i.e. GM-CSF. To insure that the extremely high level of GM-CSF expression results in only high local tissue levels of GM-CSF, the expression of the tat gene in our construct is under the control of the well-characterized inducible human heat shock protein 70B (HSP70B) promoter (29). This promoter is induced by a set of heat shock factors that respond to diverse forms of physiological and environmental stress including hyperthermia, heavy metals, oxidative stress, anti-inflammatory drugs and some toxic agents (30, 31). The tat-amplified expression of GM-CSF from this vector will thus be regulatable, with induction via a mild heat shock. Our novel heat-inducible expression vector for amplified expression of GM-CSF addresses the need for high-level GM-CSF expression, as well as providing localized GM-CSF expression via locally applied hyperthermia.

Materials and methods

Cell line and culture conditions

B16 murine melanoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in complete medium consisting of RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (Gemini Bioproducts, Calabasas, CA, USA), 100 U/ml penicillin, 100 µg/ml streptomycin, 5 ng/ml gentamycin sulfate, 1 mM non-essential amino acids, 1 mM sodium pyruvate, 0.001% (v/v) β-mercaptoethanol, and 2 mM L-glutamine. Cells were maintained at 37°C in a 5% CO_2 atmosphere. Adherent cells were detached for subculture (every 2 to 3 days) using 0.05% trypsin solution.

Lipid-mediated transfections

Cells were seeded in 6-well tissue culture plates (Falcon, Franklin Lakes, NJ, USA) at a concentration of $2x10^5$ cells/well in 2 ml complete medium. Cells were incubated at 37°C in 5% CO_2 for 24 hours until they reached 60-80% confluency. Transfection of cells with plasmids was performed 24 hours after subculture by lipid-mediated transfection (lipofection) using the lipid composition of GenePORTER2 Transfection Reagent (GTS Gene Therapy Systems Inc., San Diego, CA, USA). The lipid-DNA complex (lipoplex) preparation with GenePORTER2 was performed according to the manufacturer's protocol. Briefly, 2 µg of plasmid DNA were added to DNA diluent (GTS Gene Therapy Systems Inc.) to give a total volume of 50 µl. GenePORTER2 lipid formulation was diluted 1:5 in serum-free OptiMEM medium (GIBCO BRL, Gaithersburg, MD, USA) in a total volume of 50 µl. DNA solution and lipid mixture were combined and incubated for 5 to 10 minutes. That produced a DNA/lipid ratio of 2 µg of plasmid DNA to 5 µl of GenePORTER2 lipid. For each well, cells were washed with 2 ml PBS and subsequently incubated with 1 ml OptiMEM medium and 100 µl of the DNA/lipid mixture at 37°C in 5% CO_2. After 4 hours of exposure to the cells,

the DNA-lipid-containing transfection medium was replaced by 2 ml complete medium. Supernatants were collected 24, 48, and 72 hours after heat shock and stored at -80°C until analyzed. After each harvest medium was renewed completely by fresh complete medium.

Transfection efficiency

Transfection efficiencies were assessed 24 hours post-transfection by flow cytometric analysis of fluorescence from green fluorescence protein (GFP) after transfection with a reporter construct, expressing GFP under CMV promoter control. Cells were detached from their wells using a 0.05% trypsin solution, washed twice in PBS, and then prepared in a 1% paraformaldehyde solution for fixation before analysis. Flow cytometry was performed using a FACStar^{PLUS} flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA).

Heat shock treatment

Twenty-four hours post-transfection, cells were heat-treated in 6-well plates. Cells were washed with 2 ml PBS and 0.5 ml of complete medium was added to each well after removal of the wash buffer. This volume reduction served the purpose of minimizing heating-up periods of the suspension to favor heat shock conditions in contrast to gradual warming. Plates were sealed with parafilm and submersed in a Precision Dual Chamber water bath (Lehman Scientific, Wrightsville, PA, USA) at indicated pre-set temperatures and durations. Following heat exposure, wells were each filled up to 2 ml with complete medium and cells were returned to 37°C in a 5% CO_2 atmosphere.

GM-CSF measurement

Cell culture supernatants were collected 24, 48, and 72 hours after heat treatment as indicated. After each supernatant harvest, the remaining supernatant of each well was replaced with 2 ml

fresh complete medium. GM-CSF levels were assayed in an ELISA using the Mouse GM-CSF BD OptEIA ELISA Set (BD Biosciences Pharmingen, San Diego, CA, USA) according to the manufacturer's protocol. Dilutions were performed in Assay Diluent for OptEIA ELISA Sets (BD Biosciences Pharmingen). Reactions were performed in 96-well flat-bottomed plates for ELISA (Falcon, Franklin Lakes, NJ, USA) with the TMB Substrate Reagent Set (BD Biosciences Pharmingen). Plates were read using a 96-well microplate reader (Bio-Rad, Hercules, CA, USA).

Plasmid construction

All restriction enzymes and their buffers were obtained from New England BioLabs (Beverly, MA, USA). The GFP reporter construct was created by inserting the green fluorescence protein (GFP) gene (BD Biosciences Clontech, Palo Alto, CA, USA) into the multiple cloning site (MCS) downstream of the cytomegalovirus (CMV) promoter in pcDNA3 (Invitrogen, San Diego, CA, USA). The reporter plasmid was used to estimate the transfection efficiency of the lipid-mediated transfection by fluorescence from GFP. pcDNA3 served also as the backbone for pCMV-GM-CSF. Replacing the CMV promoter of pcDNA3 (Invitrogen, San Diego, CA, USA) by the *Bam*HI-*Hind*III fragment of the human HSP70B promoter from the p173OR plasmid (StressGen, Victoria, BC, USA) upstream of the MCS created the plasmid pH2. The functionally uncompromised *Bam*HI-*Hind*III fragment of the promoter (32), with its 451 bp in size, is significantly smaller than the full-length HSP70B promoter with 2.3 kb in size. This adaptation is advantageous for further cloning as it results in a smaller vector size. The construct pV3 originated from the pRSC plasmid, a mammalian expression vector with two multiple cloning sites (MCS) for the expression of two foreign genes, described elsewhere (33). The RSV promoter in pRSC was replaced with the HSP70B promoter fragment described earlier to generate the construct pV3. The gene for the transcription-activating factor, tat, was excised by *Xba*I digestion from pTAT, described elsewhere (34),

and ligated into the *Xba*I site of Sac-KiSS-λ (35) following *Xba*I digestion to create Sac-KiSS-TAT. The tat gene was then cut back out with *Not*I and cloned into the *Not*I site downstream of the HSP70B promoter in pV3. The CMV promoter of the original pRSC construct was replaced by the HIV2 LTR that was excised from pGL2-HIV2, described elsewhere (36), by *Bgl*II and *Hind*III digestion to produce pV3. The plasmid pV3 contains the bovine growth hormone (BGH) polyadenylation (pA) site following the MCS immediately downstream of the HIV2 promoter. The plasmids pcDNA3, pH2, and pV3, all carry Neomycin/Kanamycin (Neo/Kan) and Ampicillin selectable markers. Plasmids were isolated using a plasmid mini or maxi preparation (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Each of the backbone constructs, pcDNA3, pH2, and pV3, were digested with *Eco*RI. The 5' ends of the linearized plasmids were dephosphorylated utilizing a Shrimp Alkaline Phosphatase (SAP) kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. Ligation reactions were performed with the Rapid DNA Ligation Kit (Roche Diagnostics GmbH). A 435 bp fragment encoding the murine GM-CSF gene was excised from pUMVC1-mGMCSF (Aldevron, Fargo, ND, USA) through a digestion with *Bam*HI and *Eco*RI. The fragment was ligated into compatible sites of the MCS in the plasmid Sac-KiSS-λ, following a complete digestion of Sac-KiSS-λ with *Bam*HI and a partial digestion with *Eco*RI. The GM-CSF gene was then excised through an *Eco*RI digestion and inserted into the *Eco*RI site of the different MCSs in pH2, pV3, and pcDNA3 backbones to generate the plasmids pAD-HotAmp-GM-CSF (Figure 1A), pAD-Hot-GM-CSF (Figure 1B), and pCMV-GM-CSF (Figure 1C) respectively.

Statistical analysis

Values are given as means ± standard error of the mean (SEM). Results were compared using the Student's t-test. Statistical significance was considered at $p<0.05$.

Results

Plasmid construction

Several GM-CSF expression plasmid vectors were constructed (described in Materials and Methods): pAD-HotAmp-GM-CSF (Figure 1A), pAD-Hot-GM-CSF (Figure 1B), and pCMV-GM-CSF (Figure 1C). The minimal HSP70B promoter fragment as described in Materials and Methods is termed HSP70B promoter in the following text for simplicity. The novel vector, pAD-HotAmp-GM-CSF, contains inducible and amplifying features as two units in a single plasmid. The inducible unit is comprised of the transcription-activating factor tat located immediately downstream of the HSP70B promoter. A second promoter, the HIV2 LTR controls expression of GM-CSF. The functional binding of the tat factor to the transactivation response RNA element contained in the HIV2 LTR enhances the processivity of RNA polymerase II and/or efficiency of the elongation and elevates gene expression from basal levels (37).

GM-CSF production levels

B16 mouse melanoma cells were seeded at a concentration of 0.2×10^6 cells in 2 ml complete medium per culture well of a 6-well plate. The vectors pCMV-GM-CSF, pAD-Hot-GM-CSF, and pAD-HotAmp-GM-CSF were transiently transfected into B16 mouse melanoma cells. Negative controls were prepared as mock transfections lacking any plasmid DNA (data not shown). Cells were heat-shocked 24 hours post-transfection for the indicated temperature and duration, and returned to 37°C afterwards. The overall lipid-mediated transfection efficiency during the experiments was determined using a GFP reporter construct. According to flow-cytometric analysis, GFP positive cells ranged from 39.1 to 49.8%, with a mean of 42.2% (data not shown). Various temperatures from 39°C to 44°C were tested at 1°C increments for various time ranges of 10, 30, 60, and 90 minutes. Hyperthermia at 42°C for 30 minutes gave optimal results with highest GM-CSF levels (data not shown). GM-CSF expression levels

were dramatically decreased with exposure to temperatures of 43°C and 44°C as they caused significant cell death.

If left at 37°C, basal GM-CSF production by pAD-HotAmp-GM-CSF stayed at approximately 15% (2062 pg/ml) of its heat shock-induced amplified levels whereas no basal activity of pAD-Hot-GM-CSF was detected (Figure 2). The transient nature of GM-CSF production by the pAD-HotAmp-GM-CSF vector peaked at 24 hours followed by an exponential decrease in production. Forty-eight hours after heat induction, GM-CSF production declined to similar levels for all constructs. The decrease in CMV promoter-production expression was more gradual than with the inducible vectors. At 72 hours post-heat shock the GM-CSF production by pAD-HotAmp-GM-CSF decreases below 57% of the CMV promoter-controlled. If left at 37°C, the constitutive production by pCMV-GM-CSF and the basal production by pAD-HotAmp-GM-CSF reached similar levels during the first 24 hours and vanished to undetectable levels at later time points. As expected, cells transfected with the backbone construct of pAD-HotAmp-, lacking the GM-CSF gene, as well as mock-transfected cells, lacking any DNA, did not produce measurable amounts of GM-CSF (data not shown).

The highest GM-CSF production levels were achieved with the inducible and amplifying vector pAD-HotAmp-GM-CSF (Figure 2). Twenty-four hours after heat shock at 42°C for 30 minutes, the mean GM-CSF production by pAD-HotAmp-GM-CSF (13634 pg/ml) was over 2.5 fold higher than that of pAD-Hot-GM-CSF (5270 pg/ml). In comparison to the GM-CSF production by the strong constitutively expressing CMV promoter (4788 pg/ml), the amplified production by pAD-HotAmp-GM-CSF 24 hours post-heat shock increased over 2.8 fold.

Discussion and conclusions

The heat-inducible and amplifying elements, combined into the single multigenic construct, pAD-HotAmp-GM-CSF, responded to heat shock with the highest production of GM-CSF of all tested constructs, among which a prototypic CMV promoter construct. The inducible expression of the transactivator tat and its subsequent binding to the HIV2 LTR of the amplifying unit amplifies GM-CSF expression beyond the level that the HSP70B promoter alone could achieve. The heat-induced and amplified GM-CSF production by pAD-HotAmp-GM-CSF increased 6.6 fold over basal levels with a peak in the first 24 hours after heat shock. The rather exponential decline of production thereafter underlines the inducible and amplifying properties. However, after a transient hyperthermic induction, production levels for all vectors were expected to weaken, in addition, due to plasmid loss over time in transient transfections. The transient activity of the HSP70B promoter alone also peaked during the first 24 hours after single hyperthermic induction as previously shown by reporter gene expression (32, 38). Interestingly, constitutive GM-CSF expression by the CMV promoter in pCMV-GM-CSF was also responsive to heat treatment with expression levels close to those achieved with the HSP70B promoter in pAD-Hot-GM-CSF. A similar stress-induced increase in transcription with the CMV promoter was previously observed (39, 40).

A one-plasmid system containing the HIV2 LTR with the tat-responsive elements and the HSP70B promoter-controlled tat gene allows for uncompromised gene expression, and is more efficient than two-plasmid systems (37). A fixed ratio and coordinated expression of genes is assured in a one-plasmid system that is free from the problem of different transfection rates as occurs in co-transfection of multiple plasmids. Previously, it was shown that the incorporation of a second promoter on the same construct did not influence the temperature dependence of reporter gene expression and that in the absence of tat expression the HIV2 promoter demonstrated activity nearly independent of heat shock (38). A basal

activity of the HIV2 LTR at 37°C (in the absence of tat) may be due to the influence of other intracellular factors such as NF-κB, which are capable of transactivating this promoter.

In addition to heat exposure, the minimal HSP70B promoter fragment can be induced by gamma-radiation and the chemotherapeutic drug geldanamycin (32). However, among them, hyperthermia remained the most effective. Hyperthermia may be applied together with the classical therapies, radiation and chemotherapy. Certain chemotherapeutic agents may enhance the immune effects of genetically modified tumor vaccines (16). Therefore, a synergy effect can be achieved through the combination of classical therapies such as radiation therapy and chemotherapy in that they can additionally serve to induce the HSP70B promoter.

The vector pAD-HotAmp-GM-CSF addresses the need for very high GM-CSF production to overcome limited clinical responses with suboptimal doses. Moreover, GM-CSF production is inducible through the incorporation of the heat-responsive HSP70B promoter fragment. Thus, localized high levels of GM-CSF can be induced through localized hyperthermia. This strategy may provide an alternative to the difficult approach of locally targeted gene transfection (41-43). In an *in vivo* setting, systemic administration of the vector may then be followed by local activation, e.g. locally applied hyperthermia. The pAD-HotAmp-GM-CSF expression vector may provide a feasible strategy for locally inducible, hence improving regulation and safety in, high-level expression of GM-CSF for anti-cancer gene therapy.

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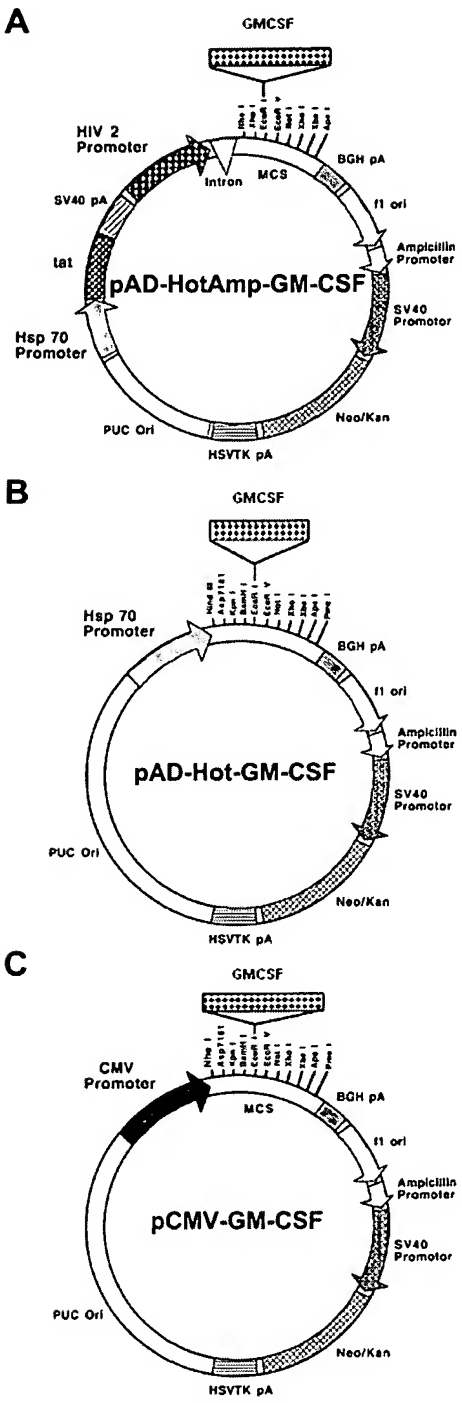
Figure legends

Figure 1. Map of the constructs. (A) The first transcriptional unit consists of the inducible HSP70B promoter that drives the expression of the first gene, the transcriptional-activating factor tat. The second transcriptional unit contains the gene encoding GM-CSF driven by the HIV2 promoter (HIV2 LTR). (B) The HSP70B promoter directly controls the GM-CSF gene expression. (C) The constitutive CMV promoter directly drives the expression of the GM-CSF gene.

Figure 2. GM-CSF production levels. Cells were heat-shocked at 42°C for 30 min 24 h post-transfection (■) or kept at 37°C as controls (□). Supernatants were harvested 24, 48, and 72 h after transfection. GM-CSF levels in the supernatants were assayed using ELISA. Each bar represents the GM-CSF production within a 24 h period. Values shown are means of triplicate experiments (± SEM). The GM-CSF levels, 24 h after heat shock, produced by pAD-HotAmp-GM-CSF were compared to pAD-Hot-GM-CSF ($p<0.001$) and pCMV-GM-CSF ($p<0.001$), and to the non heat-shocked control of pAD-HotAmp-GM-CSF ($p<0.001$)



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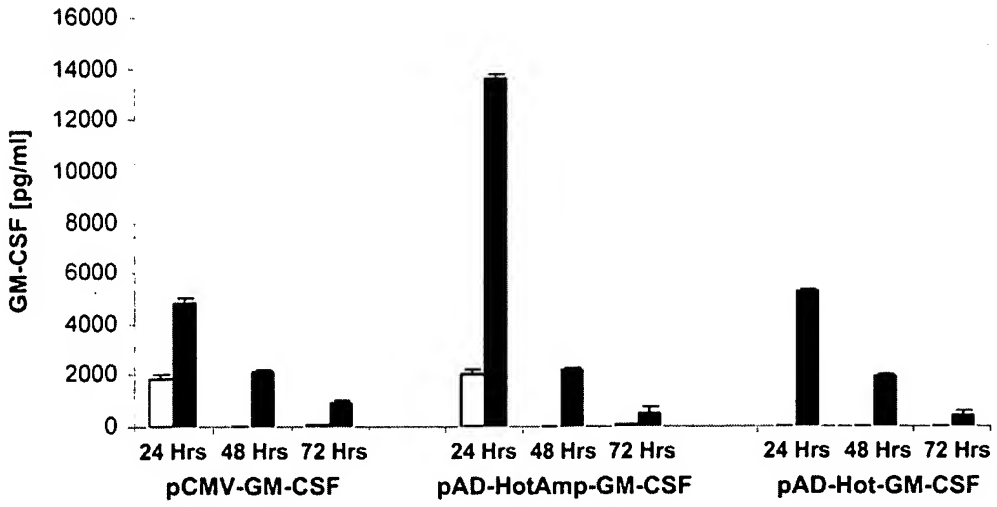

Figure 2 - Dammeyer et al.